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# Liquid-Liquid Extraction Assisted Reverse phase HPLC Method for Quantitative Estimation of Dapagliflozin from Biological Matrix-Human Plasma: Development and Validation

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**ABSTRACT**

High performance liquid chromatography (HPLC) method was developed and validated for determination of Dapagliflozin in spiked human plasma using ornidazole as an internal standard (IS) for therapeutic monitoring. The resolution was achieved on Hemsil C18 column with mobile phase composed of Methanol : Double distilled water (80 : 20%) at flow rate 1ml/min. and detection wavelength was 236 nm. Sample preparation was carried out to separate Dapagliflozin from biological matrices by liquid-liquid extraction method. The developed method could give resolved peak of Dapagliflozin and Ornidazole at 5.2 and 3.2 min. respectively. The method was validated as per ICH M10 guidelines. The method was linear in range of 10 to 50 µg/ ml and it's lower limit of quantitation and limit of quantitation were 10-50 µg/ ml respectively. The intra- and inter-day precision (%CV) were found to be in range of 0.77 to 7.20%, its accuracies were > 96% and recovery studies in range of 76.14 to 89.12%. Results of stability studies were found to be within specified range. The proposed method is characterized with highly sensitive, accurate, rapidity, as it consumes low volume of organic solvent in mobile phase and has high analysis throughput as its run time was short (~10 min).

***Keywords:*** *Antidiabetic, Bioanalytical method development, Dapagliflozin, Liquid-liquid extraction, Reverse Phase High Performance Liquid Chromatography*

## 1. INTRODUCTION

Dapagliflozin is an inhibitor of sodium-glucose co-transporter 2 (SGLT2). inhibiting SGLT2 in the proximal renal tubules, Dapagliflozin reduces glucose reabsorption and encourages its excretion in the urine. an oral antidiabetic drug called Dapagliflozin is used to treat type 2 diabetes mellitus. It comes in tablet form when it is made and sold[1]. Dapagliflozin can be estimated from bulk and its formulation using reported techniques such as tandem mass spectrometry, photodiode array detection, RP-HPLC with ultraviolet, and mass spectrometry detection[2]. In terms of sample preparation techniques (solid-phase extraction) and analysis run time, the majority of these methods were discovered to be time-consuming and expensive[3]. A review of the literature reveals that, despite the possibility of employing a straightforward sample preparation method such as liquid-liquid extraction (LLE) with UV detection, so far no RP-HPLC method for the estimation of Dapagliflozin from human plasma has been reported to date [4-8]. Therefore, our goal was to develop and verify an easy-to-use, low-cost RP-HPLC

method for estimating Dapagliflozin in human plasma. This emphasizes the necessity of creating and verifying a simple and quick bioanalytical technique, such as RP-HPLC, for the quantification of Dapagliflozin in biological materials (human plasma).the structure of Dapagliflozin is shown in fig no1.

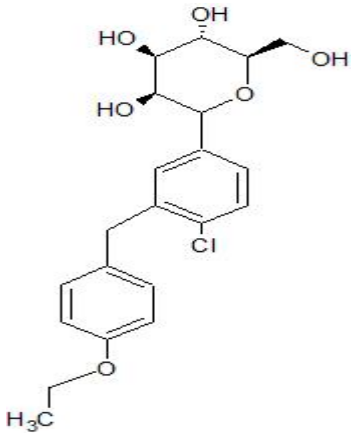


**Fig.1: Structure of Dapagliflozin[9]**

## 2. MATERIAL AND METHODS

### Chemicals and reagents

The authenticated samples of Dapagliflozin, and Ornidazole were procured along with certificate of analysis from local pharmaceutical industry, methanol (HPLC grade MeOH from Rankem), water (HPLC grade), and human plasma (Shree Sai Blood Bank, Navi Mumbai)

### Instrumentation

The Shimadzu UV- 1800 double beam UV/Vis spectrophotometer, which has a 1 nm spectral bandwidth and 1 cm thick quartz cells, was used for UV experiments. A calibrated analytical balance from LCGC was also used. Chromatographic analysis was performed using a Shimadzu UFLC (LC 2030) system with an Autosampler and an SPD-20 A prominence UV/Vis detector. The output signals were examined and processed using Lab Solutions software. Hemochrom Intsil C18–5U (4.6 mm × 250 mm), Centrifuge, Vortex (Spinix), and Ultrasonicator (INCO, India) was the analytical column used.

### Preparations of standard and Quality control (QC) sample

In HPLC grade Methanol, stock solutions of Dapagliflozin (100 µg/mL) and the internal standard (100 µg/mL) were prepared. Fresh plasma calibration standards were made by spiking drug-free human plasma matrix in solutions containing Dapagliflozin to achieve concentrations between 50 and 250 µg/mL while maintaining an IS concentration of 100 µg/mL in every sample.

### Sample preparation or extraction of plasma samples

The separation signals are impacted and the column is blocked when the plasma sample is directly injected into the HPLC system. The preparation of the sample is therefore an essential step in bioanalysis to avoid column blockage and to obtain good separation and signals. Before the chromatographic separation, the spiked Dapagliflozin is usually diluted or dissolved in an appropriate solvent to get rid of any interfering substances that could potentially help isolate and pre-concentrate the drug.

### Selection of extraction solvent

As the extraction solvent, a variety of organic solvents, including Diethyl ether, Acetonitrile, Methanol, and Ethanol, were tested and chosen based on the higher level of recovery and repeatability.

### Procedure for sample preparation

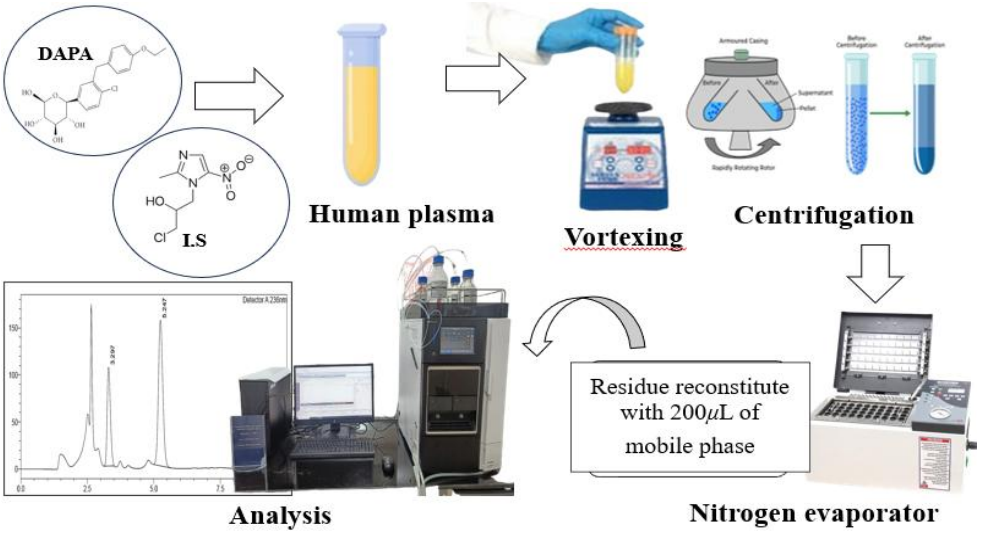


**Fig 1: Graphical presentation of sample preparation.**

The sample preparation was done by spiking blank plasma with Dapagliflozin (from 50 µL to 250 µL) and IS into a 2mL Eppendorf tube. For the precipitation of plasma extracting solvent (1000 µL) was added to this mixture. This mixture was vortexed for 5 minutes. Further, the mixture was centrifuged for 10 minutes at 2 °C at 8500 rpm. The supernatant collected was transferred into 5ml test tube and then kept that in nitrogen evaporator at 60 °C for 5 minutes then that was reconstituted diluted with 200 µL of mobile phase.

Then further HPLC analysis was carried out, where 20 µL injection volume was used for every sample as samples had different concentration levels.

**Development of RP-HPLC method and optimization of chromatographic conditions**

Numerous parameters, including sample preparation, flow rate, and mobile phase, need to be optimized in order to develop a quick, sensitive, and selective HPLC method for measuring Dapagliflozin in human plasma.

**Determination of $\lambda_{max}$**

The stock of Dapagliflozin was diluted with methanol to get the concentration of 10 µg/ml. The diluted solution of Dapagliflozin was scanned over range of 400-200 nm using a UV1800 Shimadzu double beam UV visible spectrophotometer, with methanol serving as a blank. (Quartz cuvettes were used for UV analysis having cell length of 1 cm). The UV spectra was recorded to determine $\lambda_{max}$ of Dapagliflozin.

**Selection of mobile phase**

Methanol and Acetonitrile, two organic solvents of HPLC grade, were mixed in various ratios with water using a gradient elution system to select and optimized the mobile phase for the chromatographic separation through trial and error. The optimal mobile phase was chosen based on peak shape, retention time (RT), resolution, asymmetry factor, and theoretical plate count. Dapagliflozin 10 µg/mL was injected in triplicate with a run time of 10 min after the system equilibration.

**Selection of stationary phase**

After the mobile phase, stationary phases, such as C8 and C18 columns, were used to select the column that would be used to separate and quantify Dapagliflozin from human plasma using a hit-and-trial methodology.

**Selection of internal standard**

An integral component of the bioanalytical process is the internal standard (IS). To select internal standard, various drug such as diclofenac, ibuprofen, paracetamol, ornidazole, and moxifloxacin were tried based on their chromatographic behaviour, which was similar to that of Dapagliflozin with good plasma recovery

**Optimization of the final mobile phase**

The mobile phase's composition was optimized based on the resolution, response and retention time ($R_t$) of the Dapagliflozin and IS.

**Optimization of flow rate**

In order to guarantee proper peak symmetry, stable column pressure, best possible peak resolution, acceptable retention time for both drug and IS which leads to fast analysis with best possible run time various flow rates was tried.

**Method validation**

The namely development Bioanalytical RP-HPLC method for estimation of Dapagliflozin was subjected to validation studies as per recommendations given in ICH M10 guideline[10-13]. The experimental work carried out for method validation is discussed with respective validation parameters.

**Carryover effect**

The exact percentage of IS and Dapagliflozin that the neat (blank) plasma sample carried over was calculated by injecting the blank plasma sample right away following the extraction of the highest quality control (HQC) sample. These experiments were performed in triplicate.

**Selectivity and specificity**

The developed method's selectivity and specificity were assessed by contrasting the neat plasma samples with their corresponding plasma spiked samples that came from three distinct sources, using chromatographic intercession at the $R_t$ of the Dapagliflozin and IS.

**Linearity**

To access the linearity of newly developed bioanalytical method, different volumes of working standard solution (100ppm) of Dapagliflozin were spiked in plasma so as to obtain 9 serial dilutions having concentrations of 10, 15, 20, 25, 30, 35, 40, 45, and 50 µg/ml. A 9-point calibration curve was constructed. Using the linear regression analysis, the Y-intercept, slope, and coefficient of regression ($R^2$) were determined.

**Accuracy and precision**

**Accuracy:**

Dapagliflozin was spiked in plasma samples in known amount of various concentration, like 80%, 100%, 120% of Mid quality control (MQC). The spiked samples were subsequently examined with the developed HPLC method and recovery was calculated.

The percentage of accuracy was then computed using below formula.

Accuracy (%) = (Measured concentration)/ (Nominal concentration) × 100

**Precision:**

The concentrations corresponding to lower limit quality control (LLOQ), and identified three levels of quality control samples namely, low quality control (LQC), mid quality control (MQC), and high quality control (HQC) were analysed on three different sessions of the same day with specific time interval as well as on three consecutive days which are referred as intra-day and intraday precision studies respectively.

Below mentioned formula was used to calculate the interday and intraday precision.

Precision (%CV) = SD/Mean × 100

The acceptance criteria for both accuracy and precision is within ± 15% of the actual concentration, with the exception of LLOQ where acceptance criteria for %CV is not exceeding ± 20%.

**Recovery**

A set of three QC concentrations (LQC, MQC, and HQC) were examined to ascertain the overall amount of Dapagliflozin recovered from human plasma while 100µg $mL^{-1}$ was the single concentration used to recover IS. The following formula was utilized to determine the percentage recovery:

Recovery (%) = (Peak area ratio of drug/IS extracted plasma sample)/ (Peak area ratio of drug/IS unextracted samples) ×100

**Stability**

**Freeze-thaw Stability:** Every QC sample underwent three consecutive cycles of freeze-thaw at -20°C and room temperature. The samples were then compared to newly processed samples to determine the degree of freeze-thaw stability.

**Autosampler Stability:** After being processed, QC samples were kept in auto sampler tray auto for 48 hours at 8°C. The stability of the samples was then examined.

**Short-term Stability:** Short term stability of Dapagliflozin stability in human plasma were assessed by evaluating QC samples after they were kept at -20°C for one day and three days, respectively, and compered.

**Long-term Stability:** Long term stability of Dapagliflozin stock solution QC concentrations was ascertained after it was kept for 4 hours at room temperature and for thirty days at a temperature of -20°C.

The acceptance criteria for stability studies are within ±15% of the nominal concentration.

## 3. RESULTS AND DISCUSSION

### Development and Optimization of HPLC Method for the Quantification of Dapagliflozin in Human Plasma

#### Determination of $\lambda_{max}$

The maximum absorbance of a 10 µg/mL solution of Dapagliflozin was observed at 236 nm ($\lambda_{max}$), suggesting that this wavelength is appropriate for HPLC analysis of Dapagliflozin.

#### Selection of mobile phase

To detect Dapagliflozin, a variety of mobile phases in varying organic solvent concentrations were tested, either with or without buffer solutions. The chromatogram obtained with Methanol: water (80:20 v/v) as the mobile phase composition showed a well-defined peak with a $R_t$ of 5.2 minutes.

#### Selection of stationary phase

Methanol and water were used as the mobile phase in different ratios for both C8 and C18 columns. C18 was chosen for Dapagliflozin HPLC analysis because it was found to elute the drug more quickly than the C8 column.

#### Selection of Internal Standard (IS)

The various drugs such as diclofenac, ibuprofen, paracetamol, glimepiride, ornidazole, and moxifloxacin were investigated after the mobile and stationary phases were chosen. However, ornidazole showed a symmetric peak at $R_t$ of 3.2 min, hence Ornidazole was chosen as the IS for analysis.

#### Optimization of flow rate

In order to guarantee proper peak symmetry, stable column pressure, best possible peak resolution, acceptable retention time for both drug and IS which leads to fast analysis with best possible run time as a result, 1 mL/min was the flow rate chosen for the analysis.

#### Final optimized chromatographic conditions

The final optimized chromatographic conditions needed for the analysis of Dapagliflozin are listed in Table 1.

**Table 1: Final optimized chromatographic conditions**

| Parameters | Specifications |
|---|---|
| Column | Hemochrom Intsil C 18 – 5U (4.6 mm × 250 mm) |
| Type of Method | Reverse phase |
| Mobile phase | Methanol: water (80:20 v/v) |
| Injection volume | 20µL |
| Flow rate | 1.0 mL/min |
| Detection wavelength | 236 nm |
| Run time | 10 min |
| Retention time | 5.2 min (Dapagliflozin), 3.2min (IS) |

#### Method validation

The developed method was validated as per ICH M10 guidelines and discussed below.

#### Carryover effect

No residual of Dapagliflozin and IS was carried over by the neat plasma sample.

#### Selectivity and Specificity

The retention time of Dapagliflozin and IS was found to be of approximately 5.2 and 3.2 minutes, respectively. Fig 2, Fig 3, Fig 4 and Fig 5 show chromatograms of blank human plasma, plasma spiked with IS, plasma spiked with drug and plasma spiked with IS and Dapagliflozin, respectively, with no significant interfering peaks.

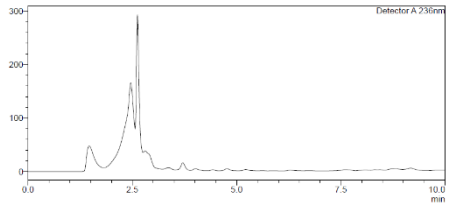


**Fig 2: Representative chromatogram of blank Human Plasma**

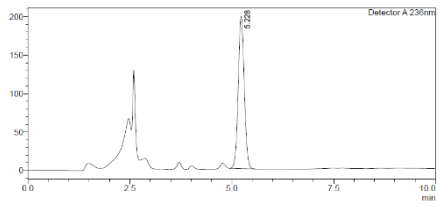


**Fig 3: Representative chromatogram of plasma spiked with drug(Dapagliflozin)**

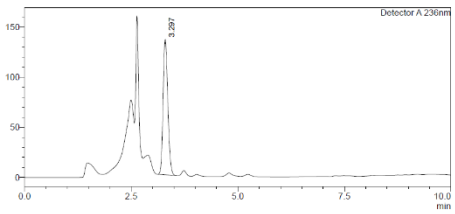


**Fig 4: Representative chromatogram of Plasma spiked with IS (Ornidazole)**

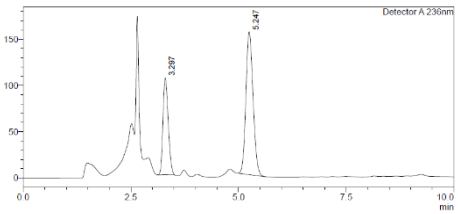


**Fig 5: Representative chromatogram Plasma spiked with drug, IS**

**Linearity**

The nine serial dilutions of Dapagliflozin (10 to 50 µg/ ml)) with fixed concentration of internal standard were prepared in plasma. Responses were recordes as peak area. The ratio of peak area of the drug to IS were ploted agains concentration to optain the calibration curve. The correlation coefficient ($R^2$) was found to be 0.997. The calibration curve of plasma spiked concentrations of Dapagliflozin is shown in Fig 6

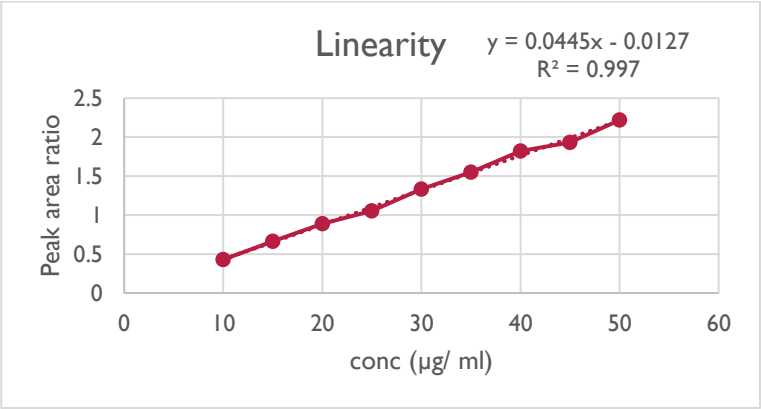


**Fig 6: Calibration curve of plasma spiked concentrations of Dapagliflozin.**

**Accuracy and precision**

**Accuracy :**

The recovery is the percentage of spiked analyte recovered and identified by the technique. A high recovery shows that the method properly measures the analyte concentration in the sample, whereas a low recovery implies potential mesurement errors. Accuracy was assessed by spiking known concentration of Dapagliflozin into plasma, with recovery ranging from 89.0 % to 91.0 % which is shown in Table 2.

**Table 2: Accuracy of Dapagliflozin**

| Sr. | Concentration (ng/ ml) | Ratio Avg | % Recovery | Overall Recovery % |
|---|---|---|---|---|
| 1 | MQC+80% | 1.555245 | 91.0 | 90.08 |
| 2 | MQC+100% | 1.6273 | 90.25 | |
| 3 | MQC+120% | 1.91424 | 89.0 | |

**Precision :**

The %CV for intra-day and inter-day precision were observed in the range of 0.772381% to 7.207878% and 2.8195 % to 5.943190% respectively (Table 3 and 4). The outcomes of precision studies were within the acceptance criteria which suggest that the developed HPLC method was extensively precise to quantify Dapagliflozin from human plasma.

**Table 3: Interday Precision of developed method**

| Within-run (Interday) precision | LLOQ (6 µg/ ml) | LQC (10 µg/ ml) | MQC (20 µg/ ml) | HQC (50 µg/ ml) |
|---|---|---|---|---|
| | | | | |
| Peak area ratio | 0.283806 | 0.3330111 | 0.633141 | 1.393629 |
| | 0.263850 | 0.3371110 | 0.641888 | 1.400656 |
| | 0.304798 | 0.3322070 | 0.629527 | 1.414899 |
| Mean | 0.284148 | 0.33411 | 0.634852 | 1.403062 |
| SD | 0.020481 | 0.002631 | 0.006356 | 0.010837 |
| %CV (%RSD) | 7.207878 | 0.787318 | 1.001148 | 0.772381 |

**Table 4: Intraday Precision of developed method**

| Between run (Intraday) precision | LLOQ (6 µg/ ml) | LQC (10 µg/ ml) | MQC (20 µg/ ml) | HQC (50 µg/ ml) |
|---|---|---|---|---|
| | | | | |
| Peak area ratio | 0.283806 | 0.333011 | 0.633141 | 1.393629 |
| | 0.276534 | 0.330575 | 0.680895 | 1.452269 |
| | 0.292544 | 0.318012 | 0.605745 | 1.472502 |
| Mean | 0.284295 | 0.327199 | 0.639927 | 1.439467 |
| SD | 0.008016 | 0.008049 | 0.038032 | 0.040965 |
| %CV (%RSD) | 2.819563 | 2.459988 | 5.943190 | 2.845845 |

**Stability :**

**Freeze thaw stability**

Three replicates each of LQC and HQC were frozen at -20°C for 24 hours. After 24 hours they were thawed unassisted at room temperature. When thawed completely, the samples were refrozen for 24 hours and the cycle was repeated. After the third freeze-thaw cycle, the samples were analyzed. The results of freeze-thaw stability are illustrtated in Table 5.

**Table 5: Freeze thaw stability of developed method**

| Freeze thaw stability | LLOQ (6 ng/ ml) | LQC (10 µg/ ml) | MQC (20 µg/ ml) | HQC (50 µg/ ml) |
|---|---|---|---|---|
| | | | | |
| Peak area ratio | 0.204014 | 0.34607 | 0.716221 | 1.8021 |
| | 0.192317 | 0.381012 | 0.703323 | 1.789978 |
| | 0.190174 | 0.358433 | 0.697378 | 1.779137 |
| Mean | 0.195502 | 0.361838 | 0.705641 | 1.790405 |
| SD | 0.007449 | 0.017718 | 0.009633 | 0.011487 |
| %CV(%RSD) | 3.810401 | 4.896664 | 1.36518 | 0.641608 |

**Autosampler stability**

Three replicates each of LQC and HQC in auto sampler at 8°C for 48 hours. The samples were then processed and analysed against calibration curve obtained along with freshly prepared calibration standards. The %CV of the determination were calculated at each QC level. The results of autosampler stability are illustrtated in Table 6 .

**Table 6: Auto sampler stability of developed method**

| Autosampler stability | LLOQ (6 µg/ ml) | LQC (10 µg/ ml) | MQC (20 µg/ ml) | HQC (50 µg/ ml) |
|---|---|---|---|---|
| | | | | |
| Peak area ratio | 0.281032 | 0.33297 | 0.633256 | 1.38921 |
| | 0.27425 | 0.328457 | 0.665827 | 1.473149 |
| | 0.292124 | 0.319416 | 0.622832 | 1.45272 |
| Mean | 0.282469 | 0.326948 | 0.640638 | 1.43836 |
| SD | 0.009023 | 0.006902 | 0.022428 | 0.043773 |
| %CV(%RSD) | 3.194476 | 2.111 | 3.500864 | 3.043256 |

**Short term stability :**

Three replicates each of LQC and HQC were kept at room temperature for three days. And then samples were subjected to analysis. The response was noted and the concentrations for each sample were back calculated from the calibration curve. The %CV of each QC level were calculated. The results of short-term stability are illustrtated in Table 7 .

**Table 7: Short term stability of developed method**

| Short term stability | LLOQ (6 µg/ ml) | LQC (10 µg/ ml) | MQC (20 µg/ ml) | HQC (50 µg/ ml) |
|---|---|---|---|---|
| | | | | |
| Peak area ratio | 0.280746 | 0.332192 | 0.634037 | 1.383081 |
| | 0.27204 | 0.341864 | 0.664532 | 1.472863 |
| | 0.290026 | 0.318562 | 0.621935 | 1.453589 |

| | | | | |
|---|---|---|---|---|
| **Mean** | 0.280937 | 0.330873 | 0.640168 | 1.436511 |
| **SD** | 0.008994 | 0.011707 | 0.02195 | 0.047265 |
| **%CV (%RSD)** | 3.201466 | 3.538191 | 3.428852 | 3.290235 |

**Long term stability :**

Three replicates each of LQC and HQC in deep freezer at -20°C for 30 days. The sampleswere then processed and analysed against calibration curve obtained along with freshly prepared calibration standards. The %CV of the determination were calculated at each QC level. The results of long-term stability are illustrtated in Table 8 .

**Table 8: Long term stability of developed method**

| **Long term stability** | **LLOQ (6 µg/ ml)** | **LQC (10 µg/ ml)** | **MQC (20 µg/ ml)** | **HQC (50 µg/ ml)** |
|---|---|---|---|---|
| | | | | |
| **Peak area ratio** | 0.280379 | 0.331015 | 0.633933 | 1.382882 |
| | 0.267055 | 0.347431 | 0.665219 | 1.473945 |
| | 0.289163 | 0.318837 | 0.620792 | 1.453814 |
| **Mean** | 0.278866 | 0.332428 | 0.639981 | 1.43688 |
| **SD** | 0.011131 | 0.014349 | 0.022822 | 0.047835 |
| **%CV(%RSD)** | 3.991571 | 4.316515 | 3.566091 | 3.329079 |

**Recovery :**

The extraction recoveries of Dapagliflozin and IS were determined by comparing the mean peak area obtained from extracted samples with un-extracted samples. The recoveries of Dapagliflozin at LQC, MQC and HQC concentration levels were found to be 76.14, 76.97 and 72% respectively. The mean recovery of IS was found to be 89.12%. Recoveries of Dapagliflozin and IS were consistent, precise and reproducible. The results of recovery studies of Dapagliflozin and IS are summarized in Table 9.

**Table 9: Results for recoveries of Dapagliflozin and IS from human plasma**

| **QC Samples** | **Mean peak area of standard solution** | **Mean peak area of extracted samples** | **Mean % recovery** |
|---|---|---|---|
| LQC | 163527 | 124517 | 76.14% |
| MQC | 419537 | 322954 | 76.97% |
| HQC | 694382 | 500011 | 72.00% |
| IS | 392761 | 350067 | 89.12% |

## 4. CONCLUSION

The present study successfully developed and validated a robust bioanalytical method for the quantification of Dapagliflozin in biological matrices, meeting the stringent requirements of international regulatory guidelines. The method demonstrated excellent sensitivity, selectivity, and reproducibility, making it highly suitable for the accurate determination of Dapagliflozin concentrations in complex biological samples.

The validation process included comprehensive assessments of critical parameters such as linearity, precision, accuracy, specificity, matrix effect, recovery, and stability under various conditions. The results confirmed that the method maintains consistent performance within the required acceptance criteria across a wide concentration range, ensuring its applicability

in diverse pharmacokinetic and pharmacodynamic studies.

Furthermore, the method's simplicity and efficiency make it an ideal candidate for high-throughput analysis in clinical and preclinical settings

Overall, this validated bioanalytical method provides a valuable analytical tool for real sample analysis of plama samples containing Dapagliflozin. It offers reliable support for applications such as drug development, therapeutic drug monitoring, bioavailability of Dapagliflozin.

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**CONFLICT OF INTEREST**

Authors have no conflict of interest.

**FUNDING SOURCE**

The work has not received any financial support.

**STATEMENT OF INFORMED CONSENT**

Not applicable.

**DATA AVAILABILITY**

The manuscript incorporates all datasets produced or examined throughout this research study.

**ETHICS STATEMENT**

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

**CLINICAL TRIAL REGISTRATION**

This research does not involve any clinical trials.

**AUTHORS' CONTRIBUTION**

Abhay Shirode:

Vineeta Khanvilkar:

Pratik Chavan:

Gauri Tamboli:

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